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The "*Rhampholeon uluguruensis* complex" (Squamata: Chamaeleonidae) and the taxonomic status of the pygmy chameleons in Tanzania

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Abstract

The specific status of several pygmy chameleons endemic to mountain massifs in the Eastern Arc Mountains, Tanzania has long been controversial due to their lack of distinctive morphological characters. In this work we extend our previous sampling of *Rieppeleon* and *Rhampholeon* species, especially from the *Rhampoleon moyeri*/*Rhampholeon uluguruensis* complex, and add data from a new mitochondrial marker to address this problem. Our results show that there is geographical structure between populations of pygmy chameleons from different mountains. This structure is especially well defined for *Rhampholeon (Rhinodigitum)*. Phylogenetic analyses confirm that both *Rh. uluguruensis* Tilbury and Emmrich, 1995 and *Rh. moyeri* Menegon, Salvidio and Tilbury, 2002 are distinct lineages, the former from the Uluguru Mountains and the latter from the Udzungwa Mountains. However, the paratype material used to erect *Rh. moyeri* belongs to a separate lineage from the holotype. Similarly, a number of additional lineages within the *Rh. moyeri*/*Rh. uluguruensis* complex recovered in the analysis may deserve specific status. At present, there is a lack of morphological characters that can be used to distinguish these lineages, suggesting that there are multiple cryptic taxa in this complex.

Key words: *Rhampholeon*, allopatric speciation, radiation, Eastern Arc Mountains, Tanzania, 12S rRNA, 16S rRNA, ND1

Introduction

Significant progress on the higher systematics of African pygmy chameleons has been made in recent years (Matthee *et al*. 2004; Mariaux & Tilbury 2006), however the recognition of species remains notoriously difficult (Tilbury 2010). Specifically, pygmy chameleons (*Rhampholeon* [sub genera *Rhampholeon* and *Rhinodigitum*] and *Rieppeleon*) have very similar external morphologies, and consequently lack sufficient external features that can be used to distinguish between species (Mariaux & Tilbury 2006; Matthee *et al*. 2004; Menegon *et al*. 2002; Rieppel & Crumly 1997). A good example of this problem is the present confusion concerning the status of *Rhampholeon uluguruensis* Tilbury and Emmrich, 1995 and *Rhampholeon moyeri* Menegon, Savidio and Tilbury, 2002 from the Eastern Arc Mountain (EAM) forests in Tanzania.

Rhampholeon uluguruensis was described on the grounds of specimens collected at Morningside on the Uluguru Mountains (Tilbury & Emmrich 1996). It was the first EAM *Rhampholeon* species identified bearing a soft, tuberculated rostral process similar to that of *Rhampholeon nchisiensis* (Loveridge 1953), which is found further south in the Southern Highlands of Tanzania and northern Malawi, and *Rhampholeon boulengeri* Steindachner, 1911 found in the montane forests of the Albertine Rift. Subsequent studies of *Rhampholeon* specimens from the Kihanga and Kitolomero valleys of the Udzungwa escarpment showed that they bore strong morphological resemblance to *Rh. uluguruensis* (Menegon *et al*. 2002) but were distinguished from it by the number of their interorbital scales and the number and arrangement of hemipenial papillae. These differences became the basis for erecting a separate species (*Rh. moyeri*, Menegon *et al*. 2002). This move appeared to be supported by nuclear and mitochondrial DNA analyses of *Rh. moyeri* specimens collected from the Kitolomero

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valley of the Udzungwa, *cf. moyeri* from the Rubeho Mountains, and *Rh*. *uluguruensis* from the Uluguru Mountains. However, denser sampling of populations of *Rh. uluguruensis*, *Rh. moyeri*, and other *Rhampholeon* populations from the EAM revealed a more complex relationship between *Rh. uluguruensis* and *Rh. moyeri*. In particular, the validity of *Rh. uluguruensis* and *Rh. moyeri* as separate species was difficult to justify given the paraphyly of both taxa, even those from the same mountain fragment (i.e. *Rh. moyeri* in the Udzungwa, Mariaux & Tilbury, 2006), and their specific status remained unresolved. Subsequent to the description of *Rh. moyeri*, several other populations of *Rhampholeon* bearing soft nose-like rostral processes were discovered through increased biological exploration of the forests scattered in the Mkungwe (close to the Uluguru), Nguu, Nguru, Ukaguru, Rubeho, and Uvidunda Mountains of the EAM (Menegon *et al*. 2003; Mariaux & Tilbury 2006; Menegon *et al*. 2008; Tilbury 2010; this paper).

The difficulty in deciphering species relationships among the genus *Rhampholeon* is not completely unexpected considering the fact that, among the Chamaeleonidae, it is particularly deficient in robust diagnostic characters; it lacks any ossified appendices, horns, or crests, the tubercles are small and often fragmented, the tail is always very short, and the head lacks well defined external features.

In the absence of robust external characters, internal soft morphological characters were previously used to distinguish *Rhampholeon* species. For example, the morphological distinction between *Rh. viridis* Mariaux and Tilbury, 2006 and *Rh. temporalis* (Matschie, 1892) was based primarily on internal hemipenial structures, and the presence of these two separate species was confirmed by molecular data (Mariaux & Tilbury 2006). By contrast, the difficulties in distinguishing *Rh. boulengeri* (from Rwanda) and *Rh. uluguruensis* (from Tanzania) based on external morphologies could not be resolved even with analysis of internal morphologies, and the 9% sequence divergence of mitochondrial markers was used to support the status of these two species (Mariaux & Tilbury 2006). Thus molecular evidence has proven to be reliable when morphology-based identifications are ambiguous.

Because the classification of *Rh. uluguruensis* and *Rh. moyeri* (the *Rh. uluguruensis*/*Rh. moyeri* complex) remains equivocal, we aimed to elucidate the phylogenetic relationship between these two putative species by analyzing molecular data from an expanded set of *Rhampholeon* specimens from new localities in previously sampled EAM mountains and from one mountain (Uvidunda) from which *Rhampholeon* specimens had not previously been collected. We also included species from the *Rieppeleon* genus in our analyses since new specimens had been collected.

We performed phylogenetic reconstruction on three mitochondrial markers using Bayesian and maximum likelihood analyses. A total of 68 individuals sampled from 12 mountain ranges were analyzed. We hypothesised that several cryptic taxa could be revealed in the phylogenetic reconstruction.

Material and methods

Specimens collection. Pygmy chameleons were collected from the EAM (Fig. 1) mostly at night and captured by hand. Specimens were kept alive in fabric bags until examination. Those needed for reference or further analysis were taken as specimens and fixed in buffered 2–4% formaldehyde, then transferred into 70% ethanol for permanent conservation. A small piece of tissue (either liver or muscle) was collected before fixation and placed in 95% ethanol for molecular analyses.

DNA Extraction and Sequencing. Approximately 20 mg of tissue was washed with sterile deionized water at room temperature for 15 minutes. DNA was extracted using the PeqGold Tissue DNA minikit (Peqlab Biotechnologies GmbH, Germany) following the manufacturer's instructions. The 12S and 16S rRNA markers were amplified by polymerase chain reaction (PCR) using primers and conditions described previously (Mariaux & Tilbury 2006; Palumbi *et al*. 1991). The ND1 gene including the 5' and 3' flanking regions was amplified with the 16dR and TMet primers described by Schmitz *et al*. (2005) and using a PCR kit containing premixed reaction reagents (Illustra, Puretaq Ready-To-Go PCR Beads, GE Healthcare, NJ, U.S.A.). The following touchdown PCR conditions were used: initial denaturation at 94°C for 3 min followed by six cycles of denaturation at 94°C for 1 min, annealing at 58°C to 53°C for 30 sec (i.e, decreasing annealing temperature by 1°C for each successive cycle), and extension at 72°C for 1 min 15 sec; 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 30 sec, and extension at 72°C for 1 min 15 sec; and a final extension at 72°C for 5 min.

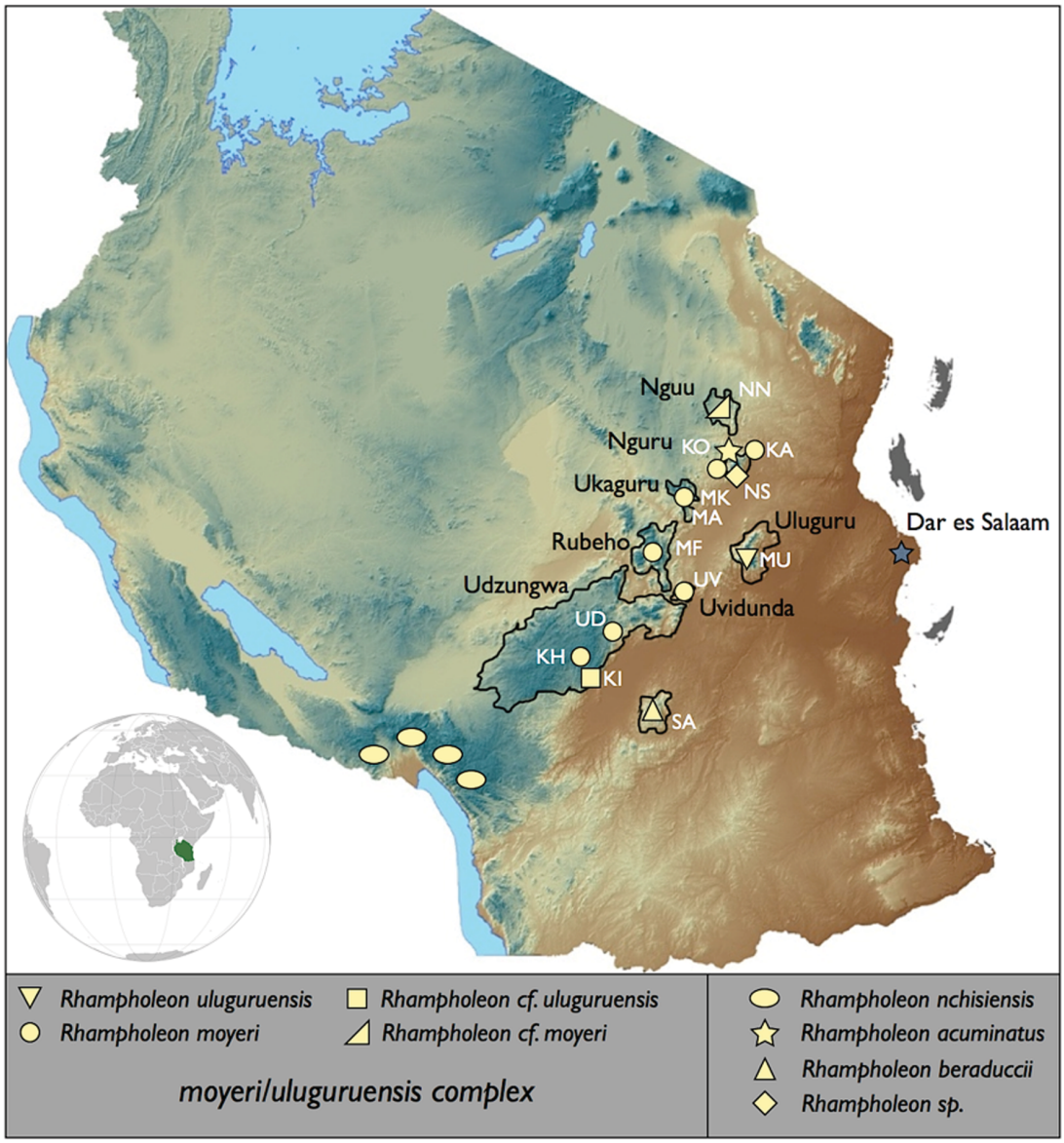

FIGURE 1. Map of Tanzania with localities (in white) and massifs (in black) where specimens were collected. KA, Kanga Forest Reserve; KH, Kihanga; KI, Kitolomero; KO, Komkore; MA, Mandenge; MF, Mafwomeru; MK, Mamiwa Kisara North Forest reserve; MU, Mkungwe; NN, Nguu North Forest Reserve: NS, Nguru South Forest Reserve; SA, Sali; UD, Udzungwa Scarp; UV, Uvidunda.

PCR products were purified using High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) and used as template in sequencing reactions with the same primers used for PCR. Sequencing was performed in-house using a BigDye sequencing reaction kit and sequenced on an ABI 377 (Applied Biosystems, CA, U.S.A.) or by an external company (Macrogen Inc. Seoul, Korea).

Molecular analyses. *Kinyongia matschiei* (Werner, 1895) 12S and 16S sequences (Mariaux & Tilbury 2006) were used as outgroup taxa for phylogenetic analyses. The ingroup consisted of 68 samples, including 27 in the *Rh. uluguruensis/Rh. moyeri* complex, for the analyses based on the rRNA sequences and a subset of these samples (n = 23), mostly in the *Rh. uluguruensis/Rh. moyeri* complex, for the analyses using the 12S, 16S, and ND1 genes. New sequences obtained in this work were deposited in Genbank with accession numbers JX301696 to JX301770 (Table 1).

Sequences were aligned using Multiple Alignment using Fast Fourier Transform (MAFFT, www.ebi.ac.uk/Tools/msa/mafft/) using default parameters, and alignments optimized manually. The 12S and 16S sequences were concatenated, and regions that could not be confidently aligned were excluded from further analysis. The ND1 coding region was translated and aligned using MAFFT (www.translatorx.co.uk). The aligned coding region was

then used to optimize the alignment of the entire ND1 region including flanking sequences. A subset of the 12S-16S sequences was concatenated with the ND1 sequence for combined analysis with all three genes.

Maximum Likelihood (ML) analysis was performed with MetaPIGA using the consensus pruning heuristic search (Helaers *et al*. 2010). The Bayesian Information Criterion (implemented in MetaPIGA) was used to identify the model of genetic evolution: GTR with gamma distribution for both the rRNA markers and the ND1 region including the coding and flanking sequences. MetaPIGA identified 15 duplicate sequences from the rRNA sequence matrix, which were removed from the analysis. The strict consensus tree and bootstrap values from 10,000 replicates are reported.

Bayesian inference (BI) analysis was performed using MrBayes version 3.1 (Ronquist & Huelsenbeck 2003) using the GTR model of evolution with gamma distribution for both the rDNA and combined 12S-16S-ND1 markers (nst = 6, rates = gamma). Default prior settings and heating schemes were used. For the 12S-16S analysis, 10,000,000 generations were run with sampling every 500 generations resulting in 20,000 samples. The 12S-16S-ND1 sequence was analyzed as above except that sampling was performed every 100 generations resulting in 100,000 samples. Two parallel runs were performed with both analyses for a total of 40,000 samples for the 12S-16S sequence and 200,000 for the 12S-16S-ND1 analysis. Runs were terminated when the average standard deviation of the split frequency fell below 0.01 and the effective sample size (ESS) was confirmed to be above 200 using the MCMC Trace Analysis Tool (Tracer v1.5.0; Rambaut & Drummond 2009). Burn-in was set at 25% and the first 5000 samples from the 12S-16S analysis and 25,000 from the 12S-16S-ND1 analysis (2.5×10^6 generations from each analysis) were discarded. The value for the burn-in was confirmed by plotting the Bayesian log likelihood (LnL) vs. time (generations) and verifying that stationary phase had been reached. The 50% majority rule consensus tree is reported.

Pair-wise genetic distances (uncorrected) were determined using PAUP* version 4.0b10 (Swofford 2002).

Results

Specimens. We analyzed a total of 69 pygmy chameleon specimens of which one was an outgroup and 68 were specimens from the EAM forests in Tanzania (Table 1). Twenty-six were new specimens from five massifs in the EAM (Table 1) and the remaining 42 specimens were from various localities in 11 montane and sub-montane regions of the EAM and were described previously (Mariaux & Tilbury 2006, Table 1 and Fig. 1). Of the new specimens, 16 were morphologically identified as belonging to the *Rh. uluguruensis/Rh. moyeri* complex, five as *Rieppeleon brachyurus* Günther, 1893, three as *Ri. brevicaudatus* (Matschie, 1892), and two as unknown *Rhampholeon* species (Table 1).

Molecular phylogenetic analysis

12S-16S rRNA. A total 941 bp (418 from the 12S and 523 from the 16S rRNA markers) were included in the matrix. Of these, 75 bp (44 bp from the 12S and 31 bp from the 16S gene) were excluded because their alignment was unreliable. The consensus trees generated using ML and BI generally gave consistent results with some minor differences in node support that did not alter the tree topology (Fig. 2). The general topology of the phylogenetic tree was concordant with what was previously reported apart from a few minor differences (Mariaux and Tilbury, 2006). It showed good support for the main *Rhampholeon* clades that had been identified, i.e. the subgenus *Rh.* (*Rhampholeon*) comprising *Rh. temporalis*, *Rh. spinosus* (Matschie, 1892), and *Rh. viridis*, and the subgenus *Rh.* (*Rhinodigitum*) comprising *Rh. nchisiensis*, *Rh. platyceps* Günther, 1893, *Rh. boulengeri, Rh. acuminatus* Mariaux and Tilbury, 2006, *Rh. beraduccii* Mariaux and Tilbury, 2006 and the *Rh. uluguruensis/Rh. moyeri* complex (Mariaux & Tilbury, 2006). Similarly, the clades within the genus *Rieppeleon* were fully concordant with those previously identified (Mariaux & Tilbury, 2006; Fig. 2).

Among the rhampholeons, the relationships of some taxa were only very weakly supported in previous analyses (Mariaux & Tilbury 2006), like the possible root position of *Rh. spectrum* in the genus or the sister taxa relationship between *Rh. temporalis* and *Rh. viridis.* These were not better supported in the present work and remain unresolved (Fig. 2).

TABLE 1. Specimens included in the study, genetic identification, and geographical origin.

Museum Number	Genetic Identification	Locality (Code*)	EAR Mountain	Accession Numbers of New Sequences			New or Previously Described
				12S rRNA	16S rRNA	ND1 rRNA	
MHNG 2609.077	*Kinyongia matschiei*						Mariaux and Tilbury 2006
MHNG 2645.001	*Rh. acuminatus*	Komkore (KO)	Nguru				Mariaux and Tilbury 2006
MHNG 2645.002	*Rh. acuminatus*	Komkore (KO)	Nguru				Mariaux and Tilbury 2006
MHNG 2655.019	*Rh. beraduccii*	Sali (SA)	Mahenge				Mariaux and Tilbury 2006
MHNG 2655.021	*Rh. beraduccii*	Sali (SA)	Mahenge				Mariaux and Tilbury 2006
ZFMK 47571	*Rh. boulengeri*	DRC					Mariaux and Tilbury 2006
ZFMK 55104	*Rh. boulengeri*	Rwanda					Mariaux and Tilbury 2006
MTSN 5092	*Rh. cf. moyeri*		Nguu			JX301737	Mariaux and Tilbury 2006
MTSN 5193	*Rh. cf. moyeri*	Nguu North FR (NN)	Nguu	JX301746	JX301720	NS	New
MTSN 5194	*Rh. cf. moyeri*	Nguu North FR (NN)	Nguu	JX301747	JX301721	JX301755	New
MTSN 5195	*Rh. cf. moyeri*	Nguu North FR (NN)	Nguu	JX301745	JX301719	NS	New
MTSN Kitolomero	*Rh. cf. uluguruensis*	Kitolomero (KI)	Udzungwa			JX301757	Mariaux and Tilbury 2006
MHNG 2624.047	*Rh. moyeri*	Mandenge (MA)	Ukaguru				Mariaux and Tilbury 2006
MHNG 2624.048	*Rh. moyeri*	Mandenge (MA)	Ukaguru				Mariaux and Tilbury 2006
MHNG 2624.056	*Rh. moyeri*	Mandenge (MA)	Ukaguru				Mariaux and Tilbury 2006
MHNG 2655.039	*Rh. moyeri*	Komkore (KO)	Nguru				Mariaux and Tilbury 2006
MHNG 2655.041	*Rh. moyeri*	Komkore (KO)	Nguru				Mariaux and Tilbury 2006
MHNG 2655.028	*Rh. moyeri*	Mafwomeru F.R. (MF)	Rubeho				Mariaux and Tilbury 2006
MTSN 5012	*Rh. moyeri*	Mafwomero F.R. (MF)	Rubeho	JX301736	JX301710	JX301759	New
MTSN 5013	*Rh. moyeri*	Mafwomero F.R. (MF)	Rubeho	JX301732	JX301706	JX301758	New
MTSN 5014	*Rh. moyeri*	Mafwomero F.R. (MF)	Rubeho	JX301733	JX301707	JX301760	New
MTSN 5592	*Rh. moyeri*	Mamiwa Kisara North F.R. (MK)	Ukaguru			JX301761	Mariaux and Tilbury 2006
MTSN 5593	*Rh. moyeri*	Mamiwa Kisara North F.R. (MK)	Ukaguru	JX301737	JX301711	JX301762	New
MTSN 7634	*Rh. moyeri*	Uvidunda (UV)	Uvidunda	JX301735	JX301709	NS	New
MTSN 8325	*Rh. moyeri*	Udzungwa Scarp (UD)	Udzungwa	JX301734	JX301708	NS	New

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TABLE 1. (Continued)

Museum Number	Genetic Identification	Locality (Code*)	EAR Mountain	Accession Numbers of New Sequences			New or Previously Described
				12S rRNA	16S rRNA	ND1 rRNA	
MTSN 8540	*Rh. moyeri*	Kanga F.R. (KA)	Nguru South	JX301743	JX301717	JX301766	New
MTSN 8541	*Rh. moyeri*	Kanga F.R. (KA)	Nguru South	JX301741	JX301715	JX301769	New
MTSN 8537	*Rh. moyeri*	Kanga F.R. (KA)	Nguru South	JX301739	JX301713	JX301764	New
MTSN 8538	*Rh. moyeri*	Kanga F.R. (KA)	Nguru South	JX301740	JX301714	JX301765	New
MTSN 8539	*Rh. moyeri*	Kanga F.R. (KA)	Nguru South	JX301742	JX301716	JX301756	New
MTSN 8542	*Rh. moyeri*	Kanga F.R. (KA)	Nguru South	JX301738	JX301712	JX301770	New
MTSN 8543	*Rh. moyeri*	Kanga F.R. (KA)	Nguru South	JX301744	JX301718	JX301763	New
MTSN Kihanga	*Rh. moyeri*	Kihanga (KH)	Udzungwa			JX301768	Mariaux and Tilbury 2006
MHNG 2624.087	*Rh. nichiensis*	Tanzania-Porotos					Mariaux and Tilbury 2006
MHNG 2664.019	*Rh. platyceps*	Mozambique					Mariaux and Tilbury 2006
MTSN 5537	*Rhampholeon sp.*	Nguru South F.R. (NS)	Nguru South	JX301730	JX301704	NS	New
MTSN 5538	*Rhampholeon sp.*	Nguru South F.R. (NS)	Nguru South	JX301731	JX301705	NS	New
MNHN 351I	*Rh. spectrum*	Cameroon					Mariaux and Tilbury 2006
MHNG 2620.032	*Rh. spinosus*		East Usambara				Mariaux and Tilbury 2006
MHNG 2620.034	*Rh. spinosus*	Mazumbai	West Usambara				Mariaux and Tilbury 2006
MHNG 2609.067	*Rh. spinosus*	Amani	East Usambara				Mariaux and Tilbury 2006
MHNG 2617.096	*Rh. temporalis*	Lutindi/Nilo	East Usambara				Mariaux and Tilbury 2006
MHNG 2617.097	*Rh. uluguruensis*	Mkungwe (MU)	Ulugurus			JX301754	Mariaux and Tilbury 2006
MHNG 2619.031	*Rh. viridis*	Chome	South Pares				Mariaux and Tilbury 2006
MHNG 2624.059	*Rh. viridis*	Kindoroko	North Pares				Mariaux and Tilbury 2006
MHNG 2624.074	*Rh. viridis*	Kindoroko	North Pares				Mariaux and Tilbury 2006
MHNG 2624.075	*Rh. viridis*	Kindoroko	North Pares				Mariaux and Tilbury 2006
MHNG 2624.082	*Ri. brachyurus*	Tanzania					Mariaux and Tilbury 2006
MTSN 5589	*Ri. brachyurus*	Mamiwa Kisara North F.R.	Ukaguru	JX301722	JX301696	JX301752	New
MTSN 5590	*Ri. brachyurus*	Mamiwa Kisara North F.R	Ukaguru			JX301753	Mariaux and Tilbury 2006
MTSN 5591	*Ri. brachyurus*	Mamiwa Kisara North F.R.	Ukaguru	JX301723	JX301697	NS	New

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TABLE 1. (Continued)

Museum Number	Genetic Identification	Locality (Code*)	EAR Mountain	Accession Numbers of New Sequences			New or Previously Described
				12S rRNA	16S rRNA	ND1 rRNA	
MTSN 8432	*Ri. brachyurus*	Nguru South (Maskati)	Nguru South	JX301724	JX301698	NS	New
MTSN 8444	*Ri. brachyurus*	Nguru South (Maskati)	Nguru South	JX301725	JX301699	JX301751	New
MTSN 8445	*Ri. brachyurus*	Nguru South (Maskati)	Nguru South	JX301726	JX301700	JX301750	New
MHNG 2609.064	*Ri. brevicaudatus*	Amani	East Usambara				Mariaux and Tilbury 2006
MHNG 2617.095	*Ri. brevicaudatus*	Tegetero	Uluguru				Mariaux and Tilbury 2006
MHNG 2617.100	*Ri. brevicaudatus*	Kihansi	Udzungwa				Mariaux and Tilbury 2006
MHNG 2619.034	*Ri. brevicaudatus*	Tegetero	Uluguru				Mariaux and Tilbury 2006
MHNG 2655.022	*Ri. brevicaudatus*	Sali	Mahenge				Mariaux and Tilbury 2006
MHNG 2655.024	*Ri. brevicaudatus*	Sali	Mahenge				Mariaux and Tilbury 2006
MHNG 2655.027	*Ri. brevicaudatus*	Sali	Mahenge				Mariaux and Tilbury 2006
MHNG 2655.030	*Ri. brevicaudatus*	Komkore	Nguru				Mariaux and Tilbury 2006
MHNG 2655.038	*Ri. brevicaudatus*	Komkore	Nguru				Mariaux and Tilbury 2006
MHNG 2655.044	*Ri. brevicaudatus*	Komkore	Nguru				Mariaux and Tilbury 2006
MTSN 5314	*Ri. brevicaudatus*	Kanga F.R.	Nguru South	JX301729	JX301703	NS	New
MTSN 8188	*Ri. brevicaudatus*	Mwanihana forest	Udzungwa	JX301728	JX301702	JX301749	New
MTSN 8226	*Ri. brevicaudatus*	Kanga F.R.	Nguru South	JX301727	JX301701	JX301748	New
MHNG 2624.078	*Ri. kerstenii*	Tanzania-Arusha					Mariaux and Tilbury 2006
MHNG 2611.010	*Ri. kerstenii*	Bred in Captivity					Mariaux and Tilbury 2006

EAR Eastern Arc Range; MHNG, Muséum d'Histoire Naturelle de Genève; MNHN, Muséum National d'Histoire Naturelle; MTSN, Museo Tridentino di Scienze Naturali; ZFMK, Zoologisches Forschungsmuseum Alexander Koenig; F.R. forest reserve; DRC, Democratic Republic of Congo; NS, not sequenced; Rh, *Rhampholeon*; Ri, *Rieppeleon*; *locality codes used in Fig. 1

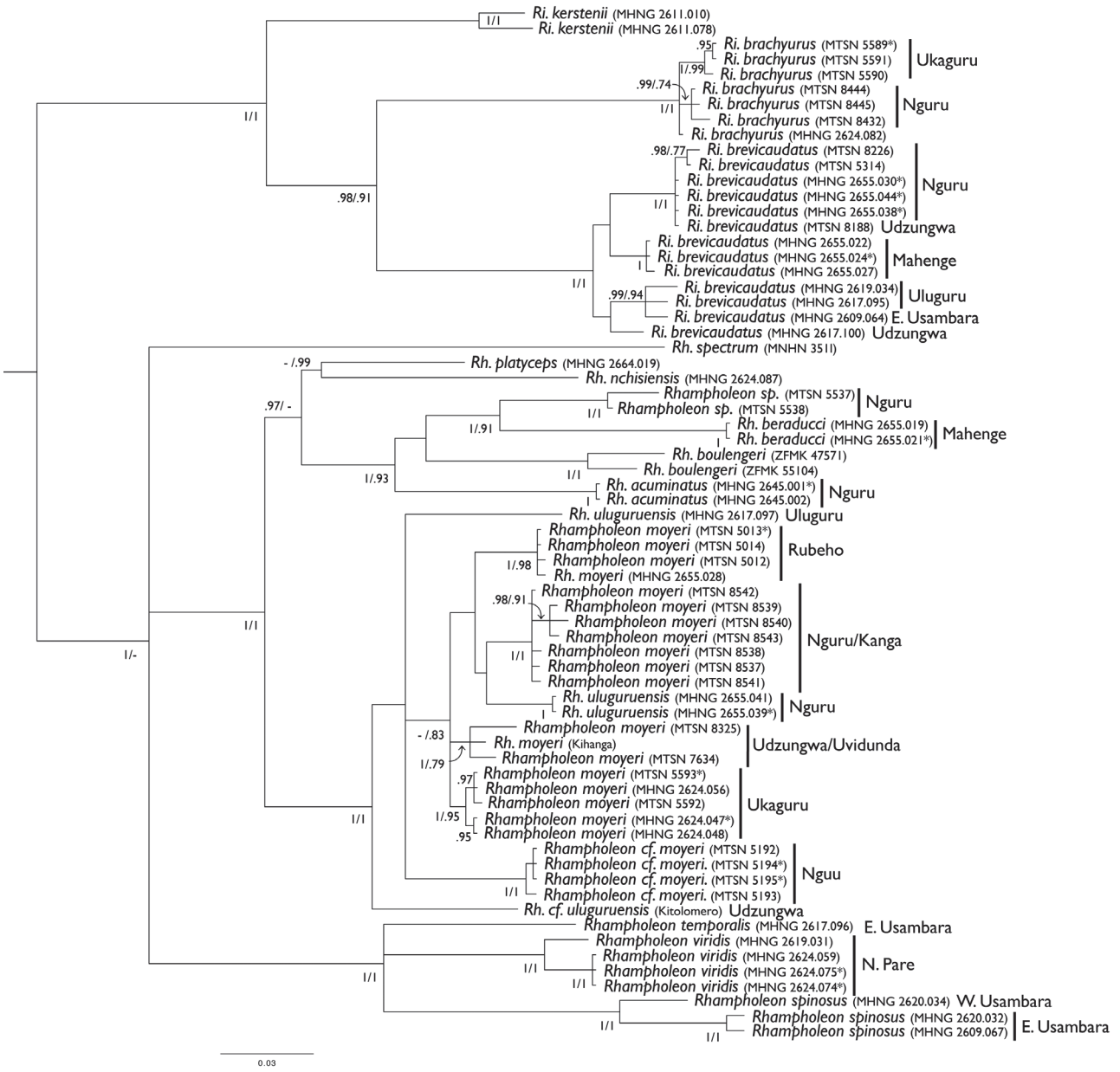

FIGURE 2. Consensus (50% majority rule) phylogenetic tree based on 12S and 16S rRNA gene analysis with Bayesian inference. Node values ≥95% for Bayesian inference and ≥70% for maximum likelihood are shown. * indicates taxa that were automatically removed by the program (MetaPIGA) in the maximum likelihood analysis because they were detected as being duplicates. The outgroup was *Kinyongia matschiei*.

The *Rh. uluguruensis*/*Rh. moyeri* complex in the 12S-16S tree was clearly supported as monophyletic by all our analyses. Overall, the clades strongly reflected their geographical origins (Nguu, Ukaguru, Udzungwa [Kihanga valley]/Uvidunda, Rubeho, Kanga, and Nguru South mountains; Fig. 2). An exception to this geographic pattern was specimens collected from two localities at different elevations in the Udzungwa Mountains: *Rh. cf. uluguruensis* from Kitolomero (at 1174 m above sea level [asl]) and *Rh. moyeri* from Kihanga (at 1780 m asl). Furthermore, individuals from the southern Nguru Mountains were divergent from those collected at Kanga, which is an outcrop of the Nguru Mountains separated from the main mountain block by a valley approximately 6 km wide.

12S-16S-ND1. To determine if the relationship between the specimens in the *Rh. uluguruensis*/*Rh. moyeri* complex could be better resolved by adding a faster evolving gene, we sequenced the ND1 gene and its flanking regions in 17 specimens from the *Rh. uluguruensis*/*Rh. moyeri* complex and in six *Rieppeleon* specimens.

Of the 1359 bp sequenced for the ND1 marker, 963 bp constituted the ND1 coding region and 423 bp the non-

protein coding flanking regions. The gene order of the sequenced region was as follows: partial 16S rRNA, tRNA-leu, ND1, tRNA-Gln, tRNA-Ile. Twenty-five bp were excluded because of ambiguous alignment in the flanking regions.

Analyses with the combined 12S-16S and ND1 sequences yielded consistent results with those from the 12S-16S sequences only (Fig. 3). Furthermore, analysis of the ND1 coding sequence only and the ND1 sequence including the coding and flanking regions gave consistent results (data not shown). Overall, the 12S-16S-ND1 trees from the ML and BI analyses (Fig. 3) were consistent with those of the 12S-16S tree (Fig. 2), despite the fact that a slightly smaller sample set was used in the analyses with the combined 12S-16S-ND1 gene: the clustering by geographical location observed in the 12S-16S tree was reproduced in the 12S-16S-ND1 analyses. However, two important differences should be noted: first, *Rh. uluguruensis* and *Rh. cf uluguruensis* Kitolomero were sister taxa on the 12S-16S-ND1 tree (Fig. 3) whereas in the 12S-16S tree *Rh. cf uluguruensis* Kitolomero was sister to the rest of the *Rh. uluguruensis/Rh. moyeri* complex (Fig. 2), though the support for the latter was weak. Second, the position of the specimens from Ukaguru and Rubeho were reversed in the two trees: whereas in the 12S-16S-ND1 tree the Ukaguru clade is sister to the Nguru clade (Fig. 3), in the 12S-16S tree it was the Rubeho clade that was sister to the Nguru clade, though this node was only weakly supported (Fig. 2).

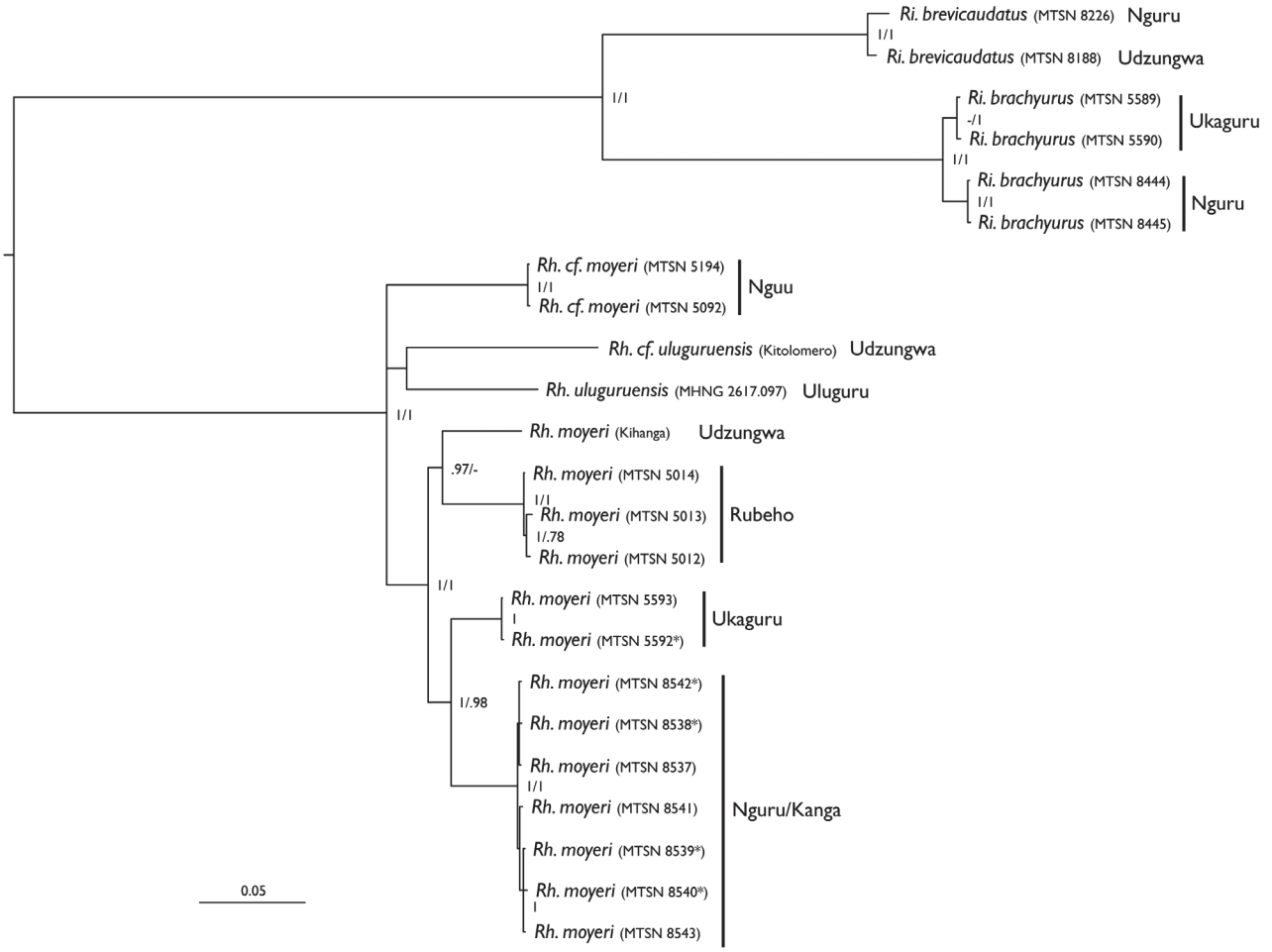

FIGURE 3. Consensus (50% majority rule) phylogenetic tree based on Bayesian and maximum likelihood analyses of the 12 and 16S rRNA genes (941 bp) and the 1359 bp region of the ND1 gene and its flanking sequences. Node values ≥95% for Bayesian inference and ≥70% for maximum likelihood are shown. * indicates taxa that were automatically removed by the program (MetaPIGA) in the maximum likelihood analysis because they were detected as being duplicates or, in *Rh. uluguruensis* MTSN 8542, as having >40% ambiguous sequences in the flanking region. The tree was mid-point rooted.

TABLE 2. Uncorrected genetic distances (minimum-maximum % divergence) based on 12S-16S rRNA (below diagonal cells) and ND1 and flanking (above diagonal cells) sequences*

Mountain (number of 12S-16S, ND1 samples)	Udz-Kitolomero (*Rh. cf. uluguruensis*)	Uluguru (*Rh. uluguruensis*)	Nguu (*Rh. cf. moyeri*)	Udz-Kihanga (*Rh. moyeri*)	Uvidunda (*Rh. moyeri*)	Rubeho (*Rh. moyeri*)	Ukaguru (*Rh. moyeri*)	Nguru-Kanga (*Rh. moyeri*)	Nguru South (*Rh. moyeri*)
Udz-Kitolomero (1, 1)	–/–	14.3	15.0–15.1	15.3	ND	14.7–15.2	13.7	14.0–14.8	ND
Uluguru (1, 1)	6.5	–/–	13.0–13.1	12.6	ND	11.7–12.2	12.7	11.9–13.3	ND
Nguu (4, 2)	6.7–6.8	6.4–6.6	0.2/ 0.0–1.1	13.2	ND	12.6–13.2	12.0	12.5–13.2	ND
Udz-Kihanga (1, 1)	6.3	5.2	5.5–5.8	–/–	ND	9.3–9.8	9.8	9.4–10.5	ND
Uvidunda (1, 0)	6.4	5.5	5.4–5.7	1.1	–/–	ND	ND	ND	ND
Rubeho (4, 3)	6.6–6.7	5.4–5.5	6.2–6.7	2.7–2.8	3.2–3.3	0.3–0.5/ 0.0–0.2	8.7–9.1	9.1–11.3	ND
Ukaguru (5, 2)	5.8–5.9	4.9–5.1	4.8–5.3	1.6–2.0	2.1–2.2	2.7–3.0	0.0/ 0.0–0.4	6.7–7.9	ND
Nguru-Kanga (7, 7)	6.6–7.4	5.5–6.1	5.7–7.3	2.9–3.6	3.0–3.8	2.8–6.6	2.5–3.5	0.0–0.2/ 0.0	ND
Nguru-South (2, 0)	7.0	4.9	6.1–6.5	3.1	3.3	3.2–3.3	2.8–3.0	2.9–3.6	ND/0.0
Udzungwa (1,0)	7.0	5.8	6.0–6.3	1.4	1.9	2.7–2.8	2.8–3.0	2.9–4.0	3.8

*Numbers in the shaded diagonal cells are ND1/12S–16S rRNA distances. Names of species in parentheses in the first row reflect the proposed nomenclature scheme. Udz, Udzungwa; ND, not determined

Species level divergences. Within the *Rh. uluguruensis/Rh. moyeri* complex, uncorrected 12S-16S rRNA sequence divergences ranged from 1.1% to 7.4% for the clades recovered (Table 2). The highest sequence divergence (7.4%) was between the specimens from Udzungwa at the Kitolomero site and the specimens from the Nguru Mountains (Table 2). With the exception of specimens from the Udzungwa Mountain, where the specimens from Kitolomero and Kihanga diverged by 6.3%, genetic distances within mountains (0.0–1.1%) were typically lower than between mountains (1.1–7.4%, Table 2). These distances were at the lower end of the values between clearly recognized *Rhampholeon* species previously reported species (7.3–15.4% Mariaux & Tilbury 2006). Interestingly, the greatest ND1 sequence divergence (15.3%) was between the two Udzungwa population of *Rh. cf. uluguruensis* (Kitolomero) and *Rh. moyeri* (Kihanga).

Comments on the genus *Rieppeleon*. The phylogenetic trees also showed some structure within the genus *Rieppeleon*, with clades reflecting geographical origin of specimens. In the Nguru Mountains, specimens from Nguru South formed a separate but related clade to those from the Kanga outcrop within the Nguru landscape, similar to that observed for the rhampholeons. The correspondence between clade and the geographical origin of *Rieppeleon* specimens was not, however, as strong as seen in the genus *Rhampholeon*. For example *Rieppeleon* species were sympatric in some of the mountains (*Ri. brachyurus* MTSN 8445, MTSN 8444, and MTSN 8432 and *Ri. brevicaudatus* MTSN 8226 and MTSN 5314 on Nguru; Fig. 2), and *Ri. brevicaudatus* that formed a monophyletic clade (MHNG 2619.034, MHNG 2617.095, and MHNG 2609.064) originated from different mountains (Uluguru and East Usambara; Fig. 2) suggesting the recent shared ancestry across riverine and coastal forests at lower elevation.

Finally, the analysis of the ND1 coding sequences also showed that the *Rhampholeon* ND1 gene had a 12-bp truncation at the 5' end (i.e. a four-amino acid N-terminal truncation of the protein). This new synapomorphy further supports Matthee *et al.*'s (2004) separation of these pygmy chameleons into two genera.

Discussion

The *Rh. uluguruensis/Rh. moyeri* complex comprises species that were previously distinguished using slight internal morphological differences but that could not be unambiguously distinguished using genetic methods (Mariaux & Tilbury 2006). In the current work, we used greater geographical sampling of populations and increased genetic information to better resolve the evolutionary relationships in the *Rh. uluguruensis*/*Rh. moyeri* group and suggest steps forward to resolve this taxonomic issue. These analyses included specimens collected from the EAM that were morphologically identified as belonging to the *Rh. uluguruensis/Rh. moyeri* complex as well as previously studied specimens. We demonstrated the presence of distinct *Rh. uluguruensis* and *Rh. moyeri* species and at least two additional candidate species. The clades including the topotypic material of *Rh. uluguruensis* and *Rh. moyeri* could be identified, allowing us to clarify the taxonomic status of these species.

Rh. uluguruensis was initially described on the grounds of specimens collected from the Uluguru Mountains only (Tilbury & Emmrich 1996) and *Rh. moyeri* based on specimens from two localities at different elevations in the Udzungwa Scarp Forest Reserve (Menegon *et al*. 2002). The holotype of *Rh. moyeri* belongs to the Kihanga population at about 1800 m asl and paratypes included specimens collected at Kitolomero 600 m. below Kihanga (Menegon *et al*. 2002). Despite some differences in body size and hemipenial papillae characters (up to 10 in the bigger Kihanga males and 12 in the smaller Kitolomero male), specimens from Kitolomero were assigned to the same taxon because of the proximity of the two sites (within about 5 km) and the overall morphological resemblance of the animals from these sites. The differences in the number of the hemipenial papillae were then considered part of the intraspecific variability. Subsequently specimens morphologically close to these two species within this complex were assigned to *Rh. uluguruensis* or *Rh. moyeri*, generally according to the collection site (i.e. Udzungwa = *moyeri*, and Uluguru = *uluguruensis*). Stations geographically close were treated as belonging to these massifs (e.g. Mahenge = Udzungwa, Loader *et al*. 2004). However molecular analyses rendered *Rh. moyeri* paraphyletic, with the Kitolomero populations closer to *Rh. uluguruensis*, and clearly separated from *Rh. moyeri* from Kihanga. This left Mariaux and Tilbury (2006) to state "We have not found any decisive argument to resolve the status of the taxa". No taxonomic changes were made by Mariaux and Tilbury (2006) who stated "no satisfactory nomenclatural system can be derived yet". The main reason why the taxonomy of *Rh. uluguruensis* and *Rh. moyeri* has been equivocal is thus due to the presence of two clearly distinct taxa within the type series of *Rh. moyeri*.

In the study by Matthee *et al*. (2004), *Rh. uluguruensis* and *Rh. moyeri* surprisingly did not form a monophyletic clade consistent with the *Rh. uluguruensis*/*Rh. moyeri* complex. We performed preliminary ML analysis (data not shown) adding the 16S sequences from the two *Rh. uluguruensis* and two *Rh. moyeri* specimens from Matthee *et al*. (2004) to our 16S matrix. In this analysis, one of Matthee *et al.* (2004) *Rh. moyeri* specimens (MTSN 001TA) was sister to *Rh. moyeri*-Kihanga (from our study), whereas their second *Rh. moyeri* specimen (MTSN 002TA) was sister to the *Rh. boulengeri* specimens from our study. We do not have a conclusive explanation for these discrepancies, especially because a re-analysis of the Matthee *et al.* (2004) matrix alone was able to reproduce the relationships between the *Rh. moyeri* samples these authors reported (Matthee *et al*. 2004). Our analysis contained 16S data from a much larger set of *Rh. uluguruensis* and *Rh. moyeri* specimens than did the Matthee *et al*. (2004) study and this may have affected the structure of this particular clade. However, we should also note that Mariaux and Tilbury (2006) previously found it difficult to distinguish *Rh. uluguruensis* and *Rh. boulengeri* morphologically, even when using soft tissues. These ambiguities could lead to taxonomic misappropriations, and we wonder whether this may be the reason that one of the *Rh. moyeri* specimens in Mathee *et al.* (2004) study formed a sister group relation with the *Rh. boulengeri* specimens in our preliminary analysis. A more thorough study including all of the *Rhampholeon* specimens from Matthee *et al*. (2004) with all of our data may help decipher these relationships. Our preliminary analyses were further limited by the fact that only the 16S data were common to both studies, and future work should include more genes for comparison.

Based on these results, we propose that *Rh. uluguruensis* from the Uluguru Mountains be considered a species restricted to the Uluguru only and having a genetic divergence ≥5% in the 12S and 16S genes compared with all other known *Rhampholeon* species. In the Udzungwa Mountains there are two highly divergent monophyletic clades, both currently assigned to *Rh. moyeri* and part of a type series. The morphological differences (such as the differences in numbers of hemipenial papillae and in body size) between the individuals coming from the two localities in the Udzungwa Mountains, also noted in the original description (Menegon *et al*. 2002), are supported by a genetic difference of 6.3% in the 12S-16S sequences. We suggest, therefore, that the *Rh. moyeri* population from Kitolomero be considered endemic to this Valley and should be provisionally designated *Rh. cf. uluguruensis* given its phylogenetic proximity to the Uluguru specimen (*Rh. uluguruensis*). Thus, *Rh. moyeri* from Kihanga remains valid but with the paratype materials from Kitolomero (MTSN 001TA, MTSN 002TA, and MTSN 003TA in Menegon *et al*. 2002) removed from the description, given the paraphyletic status of these populations. The status of the fourth paratype from Kitolomero (NMZB 11579) was not evaluated in this study but presumably also belongs to *Rh. cf. uluguruensis* and is removed from the *Rh. moyeri* type series as well.

The fourth, highly divergent monophyletic clade proposed as the second candidate species is represented by the population in the Nguu Mountains, provisionally considered *Rh. cf. moyeri*, and has genetic divergence (based on 12S-16S rRNA sequences) from topotypical *Rh. moyeri* and *Rh. uluguruensis* of 5.5–5.8% and 6.4–6.6%, respectively. The taxonomic position of this species was briefly discussed by Menegon *et al*. (2003). Though these are likely to deserve a specific status, we do not consider it appropriate to take any nomenclatural decision until suitable morphological characters are defined and detailed molecular analyses (including nuclear genes) are completed. The remaining samples from the *Rh. uluguruensis/Rh. moyeri* complex all have genetic similarity between 1.1% and 3.6% with *Rh. moyeri* and between 4.9% and 6.1% with *Rh. uluguruensis*. We propose a conservative approach and consider all of these as belonging to the *Rh. moyeri* complex given their closer genetic affinities. However, the existence of additional cryptic taxa in the complex, particularly from Nguu, Nguru, Rubeho, and Ukaguru is likely.

Most of the divergent lineages of *Rhampholeon* were endemic to single mountain blocks despite short geographic distances between them, and this apparently followed a one species-one mountain scheme similar to other taxa with low vagility (e.g. Turner & Channing 2008; Tolley *et al*. 2010). This is probably due to a long history of isolation in forest fragments, similar to chameleons in the genus *Kinyongia* (Tolley *et al*. 2011; Mariaux *et al*. 2008), which occur in the same forest fragments as the *Rhampholeon* species in our study. Despite the large genetic differentiation between the candidate species, the phenotypes are extremely similar and particularly difficult to distinguish using traditional morphological characters. Given the apparent morphological similarity across this genus, it is likely that similar environmental pressures constrain their morphology, resulting in a strongly conserved phenotype (Losos, 2008). However, further studies that include examination of ecological similarities, as well as investigation of ecologically relevant morphological traits would be required.

It is interesting to note, however, that the phylogenetic relationships did not always reflect a pattern of close

geographical proximity between sister groups. For example the "Nguu clade" and "Nguru clade" were clearly separated despite both being located in the northern mountain chain considered here (Fig. 2). On the other hand, in the 12S-16S analysis, the "Rubeho clade" and the "Nguru clade" (Fig. 2) were grouped on the tree whereas these massifs are not geographically the closest. Some of these observations need to be cautiously interpreted because of the relatively poor support of the internal nodes within the *Rh. uluguruensis/Rh. moyeri* complex. In the latter case the addition of ND1 in the analysis resulted in a better supported and more logical relationship, with the "Rubeho clade" being related to *Rh. moyeri* from the geographically close Udzungwa (Fig. 3).

Among the samples analyzed, two specimens (*Rhampholeon sp*., Fig. 2) collected at high altitude in the Nguru Mountains fell outside the *Rh. uluguruensis/Rh. moyeri* complex (>10% divergence in the 12S and 16S rRNA genes compared with the specimens in the *Rh. uluguruensis/Rh. moyeri* complex). They are sister to *Rh. beraduccii* and belong to an undescribed taxon. They show close morphological resemblance with both *Rh. beraduccii* and taxa in the *Rh. uluguruensis/Rh. moyeri* complex but they differ from both by the presence of a wide and conspicuous scaly structure at the base of the rostral process where the canthi rostralis meet.

Unlike the rhampholeons, our different *Rieppeleon* samples could be attributed to well-defined, and monophyletic taxa. Interestingly however, our numerous *Ri. brevicaudatus* samples did not fully group according to their geographical origin. Although specimens from a specific mountain belonged to a single clade, the structure observed with rhampholeons was not fully replicated and, in a few instances, rieppeleons from distant massifs, like the Eastern Usambara and the Uluguru, were found in single clades (Fig. 2). This may be because the low altitude habitat of these species, which consists of relatively recent forested connections, prevents the isolation of separated lineages.

The substantial molecular differences among the rhampholeons observed in the current study are not supported by the morphological characters previously analyzed in the description of these taxa and populations. Incongruence between morphological and molecular characters (i.e. when morphologically similar species are not necessarily phylogenetically close relatives) is well known in chameleons. For example, the recently described *Trioceros nyirit* Stipala *et al*. 2011 is sister with the morphologically dissimilar *T. schubotzi* (Sternfeld, 1912), while the morphologically more similar *T. hoehnelii* (Steindachner, 1891) is much more distantly related (Stipala *et al*. 2011).

The expected high level of micro-endemism exhibited across the EAM in the subgenus *Rh.* (*Rhinodigitum*) has been observed in other chameleon genera (Glaw *et al*. 2012; Tolley *et al*. 2011) as well as in other groups. In particular, a similar situation is known for several groups of amphibians occurring across the Eastern Arc Mountains (Loader *et al*. 2011; Menegon *et al*. 2011; Lawson 2010). Interestingly for amphibians, high-levels of endemism are seen in the Udzungwa where several species confined to valleys or forest patches are known (Clarke 1988; Poynton *et al*. 1998). Kitolomero valley, the only known site for the *Rh. cf. uluguruensis* is also the only known site for the toad *Nectophrynoides poyntoni* Menegon, Salvidio and Loader, 2004 and the whole Udzungwa Scarp Forest Reserve is home to several highly restricted species, like *Nectophrynoides wendyae* Clarke, 1988, *Nectophrynoides asperginis* Poynton, Howell, Clarke and Lovett, 1994 (Menegon *et al*. 2004), and *Hyperolius kihangensis* Schiøtz and Westerfaard, 1999 (Schiøtz 1999). The presence of two distinct species within relatively short distances in this region is therefore seemingly not uncommon – as shown in *Rhinodigitum*.

In conclusion, our genetic analyses demonstrate that the *Rh. uluguruensis/ Rh. moyeri* complex consists of two distinct species that arose in distinct localities or mountains. At least two additional species (*Rh. cf. moyeri* from Nguu Mountains and *Rh. cf. uluguruensis* from the Udzungwa Mountains at the Kitolomero site), and potentially more, may be defined in the future when more diagnostic features can be defined. The critical value of each of the remaining Eastern Arc forests in term of biodiversity and their priority status for conservation is thus once more emphasized.

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